454A

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8865

Application For Renewal of Research Graht

(Use extra pages as needed)

First Renewal ☒ Second Renewal ☐ Date: 1/20/75

1. Principal Investigator (give title and degrees):

Allen B. Cohen, M.D., Ph.D., Associate Professor of Medicine

2. Institution & address:

Temple University
Health Sciences Center
3420 North Broad Street
Philadelphia, Pennsylvania 19140

3. Department(s) where research will be done or collaboration provided:

Department of Medicine

4. Short title of study:

The Genetic Defect in Alpha-1-antitrypsin Deficient Patients

5. Proposed renewal date: July 1, 1975-Grant for year 2

6. How results to date have changed earlier specific research aims:

See appended Sheets

7. How results to date have changed earlier working hypothesis:

Results to date have not changed the earlier working hypothesis.

8. Any additional facilities now required? Describe briefly:

New facilities at Temple University include about 1,000 sq. ft. of newly renovated laboratory space. This space represents about a 30% increase over my existing facilities at University of California. All of my equipment can be moved with me to Temple University and no additional facilities will be required.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

Mrs. Dagmar Geczy, my chief laboratory technician will go with me to Temple University. This should minimize the transition time.

Dr. Lo will not move to Temple U. In her place, Dr. Richard Turner will join me at Temple. Dr. Turner did his Ph.D. at the University of Minn. and a post-doctoral fellowship at this institution with Dr. Irving Liener, a well known research scientist in the area of protein inhibitors of proteolytic enzymes in plant systems. Therefore, Dr. Turner is admirably suited to continue the work on AAT. Bibliography is appended.

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

Abstracts:

Lo, T.W. and Cohen, A.B.: The Interaction between alpha-1-antitrypsin and sepharose-bound elastase. In Press. Fed. Proc. Accepted for presentation at the American Society of Biological Chemists, national meeting, Atlantic City, May 15, 1975.

Cohen, A.B.: A Biochemical Approach to Human Emphysema. Submitted to the American Lung Association for consideration for presentation at their annual meeting in Montreal, May 18, 1975. Will be publised in the American Review of Respiratory Diseases if accepted for publication.

REVIEW: Cohen, A.B. Ch. 8. Alpha-1-antitrypsin: A systemic Determinant of Lung Structure and Function. In the Biochemical Basis of Pulmonary Function, Ed. R. Crystal. A volume in the series: "Lung Biology in Health and Disease", Ed.C. Lenfant. Marcell Dekker, Inc., New York, In Press.

12. Summary progress report (append in standard form as separate document, unless recently submitted).

See page

13. Budget for the coming year: 7/1/ 75-6/30/76

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Allen B. Cohen	30%	
Richard Turner (Base $12,000)	100%	REDACTED
Technical		
Mrs. Dagman Geczy (Base $16,100)	100%	REDACTED
(Salaries include 15% fringe benefits)		
		REDACTED
Sub-Total for A		

B. Consumable supplies (by major categories)

Enzymes	$2050	Ampholytes (25 runs)	$ 550
Substrates	$1550	General Chemicals	$1585
Column resins	$ 850	Pipettes (Vol. & Micro)	$ 550
Glassware	$1050	Isotopes	$ 750
Miscellaneous laboratory and office supplies			$1250

Sub-Total for B $10,185

C. Other expenses (itemize)

Animals for immunization	$3445
Blood for alpha-1-antitrypsin preps (200 units)	$5145
Publication costs (reprints and page charges)	$1645
Service contracts on equipment	$2145
Travel ($500 for each professional)	$1000

Sub-Total for C $13,380

Running Total of A + B + C $55,880

D: Permanent equipment (itemize)

Miscellaneous items of equipment. This will probably include another power supply and another polyacrylamide disc gel electrophoresis apparatus since our current equipment is overutilized.

Sub-Total for D $ 1,500

E. Indirect costs (15% of A+B+C) E $ 8,382

Total request $65,762

13. Budget for the coming year:

BUDGET COMMENTARY

For First Renewal - July, 1975

1. As shown on page 4 of the application, a grant request submitted to the National Heart and Lung Institute is pending. The grant is similar to this application but would start in September, 1975. If both grants are funded to the extent requested, the 1976 renewal of the Tobacco Research Council grant will not be requested, and the balance of this first renewal following the starting date of the NHLI grant will be returned to the Council.

Application for a supplemental grant from the Council for Tobacco Research for two months (May-June, 1975) for $5,326 has been requested through the University of California,San Francisco, at the request of Dr. Robert Hockett, and this request is pending.

2. The salary for the Ph.D. position (Dr.Richard Turner) was budgeted on the original grant request.

3. Items in budget categories B and C have been computed by adding 5% to the request for the first year.

14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None after July 1, 1975			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
A Biochemical Approach to Human Emphysema	National Heart and Lung Institute	140,575 (year 1)	September, 1975-1980
(See budget Commentary, item #1 regarding this grant)			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal Investigator

Typed Name: Allen B. Cohen, M.D., PH.D.

Signature: [signature] Date: 1-17-75

Telephone: (415) 648-8200 ext. 273 or 627
(Area Code) (Number) (Extension)

Checks payable to

Temple University

Mailing address for check:

Mr. David W. Siegel, Assoc. Vice Pres. Fin.
Temple Univ. Health Sciences Center
~~3120 North Broad St., Philadelphia, Pa. 19140~~

Responsible officer of institution:

Typed Name: Dr. Samuel S. Herman

Title: Assoc. Vice President, Research Administration

Signature: [signature] Date: 1/25/75

Telephone: REDACTED
(Area Code) (Extension)

10035462236

6. How results to date have changed earlier specific research aims:

Our original plan required the isolation of alpha-1-antitrypsin from patients with a genetically determined deficiency of AAT in their blood. Since this abnormal protein occurs in low concentrations and is very unstable in patients with this disease, conventional means of isolation of the protein have not been successful. One of the approaches which we have employed to isolate the abnormal AAT is based on the knowledge that AAT binds to various proteolytic enzymes. There are only two elastase inhibitors in human serum. One of them is AAT and the other is more than 20 times heavier than AAT. Therefore, an elastase affinity column was thought to be a logical approach to the isolation of AAT. The details of the experiments are presented in the progress report. In brief, the AAT which was washed from the elastase column was no longer an enzyme inhibitor and it had lost a peptide of about 4,000 daltons molecular weight. When the interaction between isolated AAT and elastase was then studied in vitro with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) it was evident that AAT exhibited similar behavior in solution. These results made it evident that while the elastase affinity column could not be used to isolate abnormal AAT for structural studies, the elastase on the column might have cleaved AAT at the inhibitory site and offered a convenient method for determining the amino acid residue at the inhibitory site. Since this information is the key to developing reagents which might replace AAT in patients with a deficiency of this serum protein, we would like to pursue the study of the elastase inhibitory site with the affinity column. In addition, the techniques developed during this study may permit us to identify the key amino acids at the other enzyme inhibitory sites on AAT. Other lines of investigation of the inhibitory sites of AAT in progress in our laboratory will permit us to confirm the data from the affinity column by other means. These experiments are alluded to in the progress report.

9. Biographical sketch of new key personnel:

Curriculum Vitae

Personal: name Richard H. Turner

REDACTED

current address

REDACTED

office phone 1-612-3-3-1281

marital status REDACTED

Extra-professional interests:

foreign language and travel fluent in German and Spanish reading knowledge of French extensively travelled throughout Western Europe, Canada and Mexico

music proficiency in classical violin and fiddle concert performances with the Minneapolis and St. Paul Civic Orchestras

business economics portfolio manager and co-trustees of the James E. Turner Trust Fund director on the board of Engineering Products Co. of Waukesha, Wisconsin engaged in trading stocks and bonds on the New York Stock Exchange and options on the Chicago Board of Options

counselor for Project Motivation an organization dealing with disadvantaged children in the Twin Cities

sports intramural atheletics

Honors:

NIH trainee 1968-73

postdoctoral training under a grant from Proctor and Gamble 1974

recipient of the Wisconsin State German Teachers Competition The first prize was a six-week cultural exchange with German families in Bonn, Vienna, and Berlin.

Academic Training:

B.A. Carleton College 1968 major biology

Ph.D. University of Minnesota major biochemistry minor physical and organic chemistry

In addition to coursework in the major and minor fields, the traineeship provided the opportunity to teach at both the undergraduate and graduate levels. This included organizing and preparing laboratory exercises and a limited number of lectures.

Research interests: My thesis work involved the isolation and characterization of soybean proteins by classical methods. The plant protein proteinase inhibitors which were isolated were derivatized by a number of organic reactions. The interaction of the inhibitors with proteases was characterized with regard to their thermodynamics and kinetics. This aspect of the thesis will soon be submitted to Biochemistry for publication.

My current research is involved with the high speed isolation of plant lectins and detoxification of soy flour by chromatographic procedures. The purified lectins will be used to probe changes in malignant cell membrane structure.

10035462 28

10. Plan for the Coming Year:

From the work in our laboratory during the past year, a hypothesis can be proposed for the biochemical mechanism of AAT and the hypothesis can be subjected to experimental tests. Earlier, we proposed that AAT interacted with enzymes in a manner similar to their interaction with protein substrates but instead of cleaving the substrate, AAT and the enzyme formed a stable intermediate. The interaction of serine proteases and substrates has been schematized by Stroud (Fig. 10-1). The schematic shows the different intermediates which develop during the interaction of serine proteases with substrates. Recently, Moroi (Biochem. Biophys. Acta. 359:130, 1974) has suggested that the AAT-enzyme complex is one of these intermediates, called the acyl intermediate (Panel 4, Fig. 10-1). In this intermediate the peptide on the amino-group side of the peptide cleavate has fallen from the complex and the serine hydroxyl at the enzyme catalytic site is bound to the carboxyl group of the amino acid at the end of the remaining protein fragment. This bond is an ester bond, and as mentioned in the progress report these bonds are often susceptible to cleavage under mild alkaline conditions. Our work in the past year has shown that the AAT in the AAT-enzyme complex has already lost a peptide and that the loss of the peptide is not dependent on unbound or active trypsin in the solution. In addition, the bond between the AAT and trypsin is broken under mild alkaline conditions. These data are highly suggestive that the AAT-enzyme complex is indeed an acyl intermediate (Fig. 10-2).

During the ensuing year we wish to test the next step in the hypothesis: Enzymes are attached by an alkaline labile bond to the new carboxy terminal amino acid on AAT. The design of the experiments are diagramed in Figure 10-3.

We have already determined that KOH can cleave the AAT-trypsin complex into an active enzyme and a fragment of AAT with a small peptide missing. If the enzyme is bound to the carboxy-terminal amino acid through its carboxyl group, as it must be to fulfill the requirements of the hypothesis, then the AAT in the complex will have no chemically recognizable carboxy-terminal amino acid. Carboxypeptidases are enzymes which cleave amino acids from the carboxy-terminal end of proteins, one residue at a time. Therefore, if the AAT-enzyme complex is an acyl intermediate and if a carboxypeptidase of appropriate specificity is added to the complex, the carboxy-terminal amino acid of the enzyme-bound fragment of AAT will not be removed (Fig. 10-3, left arrows). However, if the complex if first dissociated and then the carboxypeptidase is added, the amino acid at the enzyme inhibitory site will be removed and determined in the amino acid analyzer (Fig. 10-3 right arrows).

The experiment can be further illustrated by describing its use in determining the amino acid at the inhibitory site on AAT for trypsin. Carboxypeptidase B (CPB) is an enzyme which cleaves arginine and lysine amino acids from the carboxy-terminal end of proteins and trypsin cleaves proteins at arginine and lysine residues inside of the peptide chain. Therefore, a solution containing AAT-trypsin complexes will be divided into two aliquots and one aliquot will be dissociated with KOH. Then both aliquots will be treated with CPB. Amino acids which are released will be analyzed on an amino acid analyzer. If trypsin is bound to carboxy-terminal arginine or lysine on the remaining AAT peptide, then an arginine or lysine should be removed only from the complex dissociated in KOH. This experiment would have the dual function of demonstrating that trypsin is bound to the carboxy-terminal amino acid of the remaining peptide, and identifying this key amino acid. All enzymes inhibited by this

site on AAT would have to be able to cleave proteins at this same amino acid; therefore, if an inhibitor were designed by starting with this amino acid and adding a binding group, then we could say with certainty that the resultant inhibitor had the same function as the AAT inhibitory site which it was designed to mimic.

Other inhibitory sites on AAT may be identifiable in the same manner.

Significance of the Research:

Individuals with a severe deficiency of AAT in plasma have a high incidence of emphysema. Since AAT is known to be an enzyme inhibitor, emphysema in such patients might logically be attributed to the unrestrained action of an enzyme or several enzymes on pulmonary tissues. A chemical substitute for the missing inhibitor is of obvious clinical importance. If the hypothesis for the biochemical mechanism of action of AAT presented in this protocal is correct, it may provide a basis for developing drugs that block enzymes inhibited by AAT and prevent the development of emphysema in deficient patients. In addition, if an excess of similar enzymes, not blocked by normal levels of inhibitor, is found to cause other types of emphysema, then the drugs may be useful in preventing emphysema in patients other than those with a deficiency of AAT.

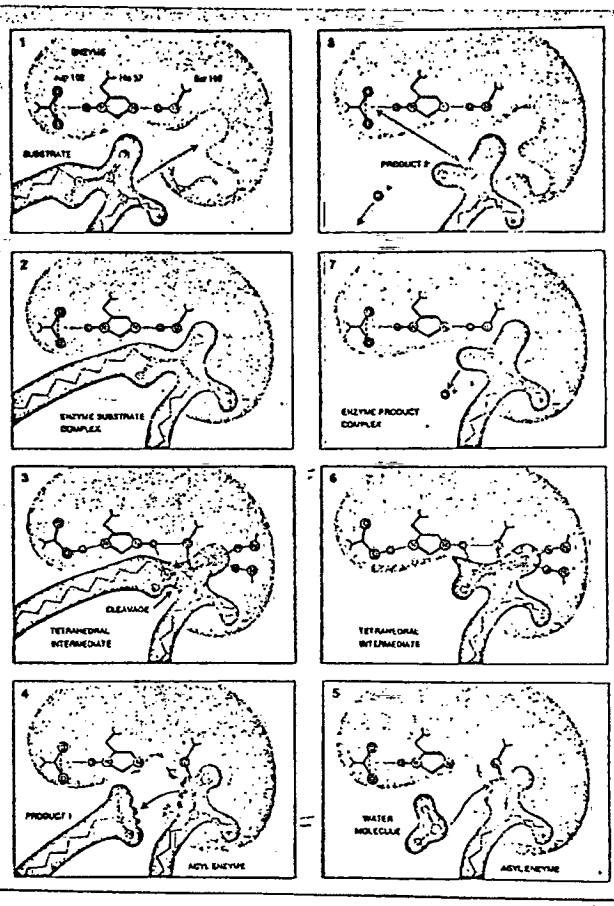
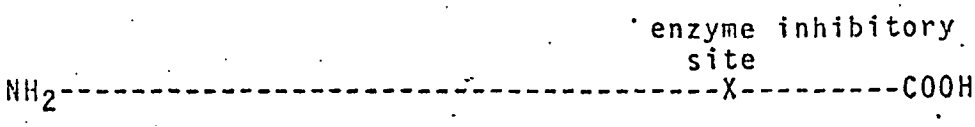


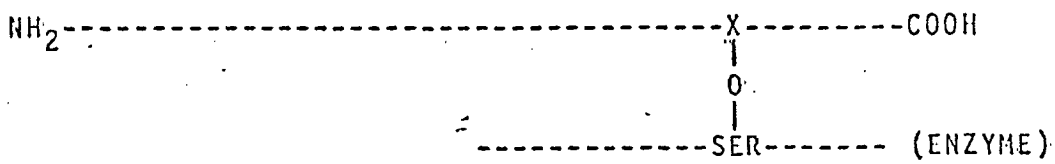
Figure 10-1:
CATALYTIC MECHANISM of trypsin and other serine proteases accelerates the hydrolysis of peptide bonds by providing intermediate states for the reaction and by smoothing the transition from one intermediate to the next. Enzyme and substrate must first come together (1) to form a precisely oriented complex (2). The oxygen atom of serine 195 then bonds covalently to the substrate carbon, forming a tetrahedral intermediate (3); the proton, or hydrogen ion, from serine 195 is transferred to the substrate nitrogen. The proton transfer breaks the peptide bond, and the first product is liberated (4). The remaining complex is called an acyl enzyme; it breaks down to regenerate free enzyme in steps that are symmetrical with those of the first half of the process. A water molecule enters the reaction (5), its hydroxyl group forming with the substrate another tetrahedral intermediate (6). The hydrogen ion from the water molecule is transferred to serine 195, breaking the covalent bond between enxyme and substrate (7). The second product is now freed (8); its departure is hastened by repulsion between negatively charged carboxyl groups of product and aspartic acid 102. Histidine 57 and aspartic acid 102 participate in the proton transfers.
(R.M.Stroud, Sci.Am., 237:74, 1974)

1. ALPHA-1-ANTITRYPSIN



2. ALPHA-1-ANTITRYPSIN-ENZYME COMPLEX

(Tetrahedral Intermediate)



3. ALPHA-1-ANTITRYPSIN-ENZYME COMPLEX

(Acyl Intermediate)

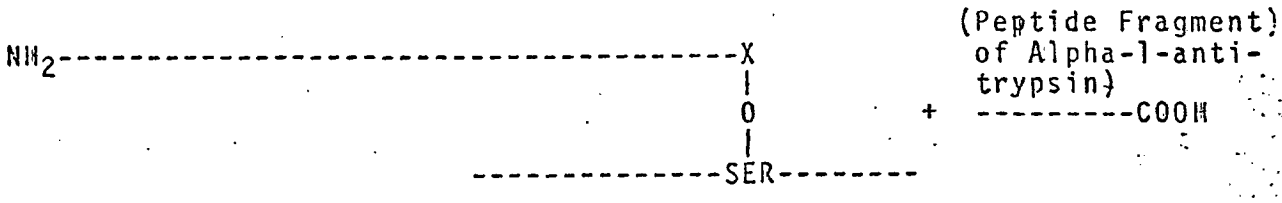
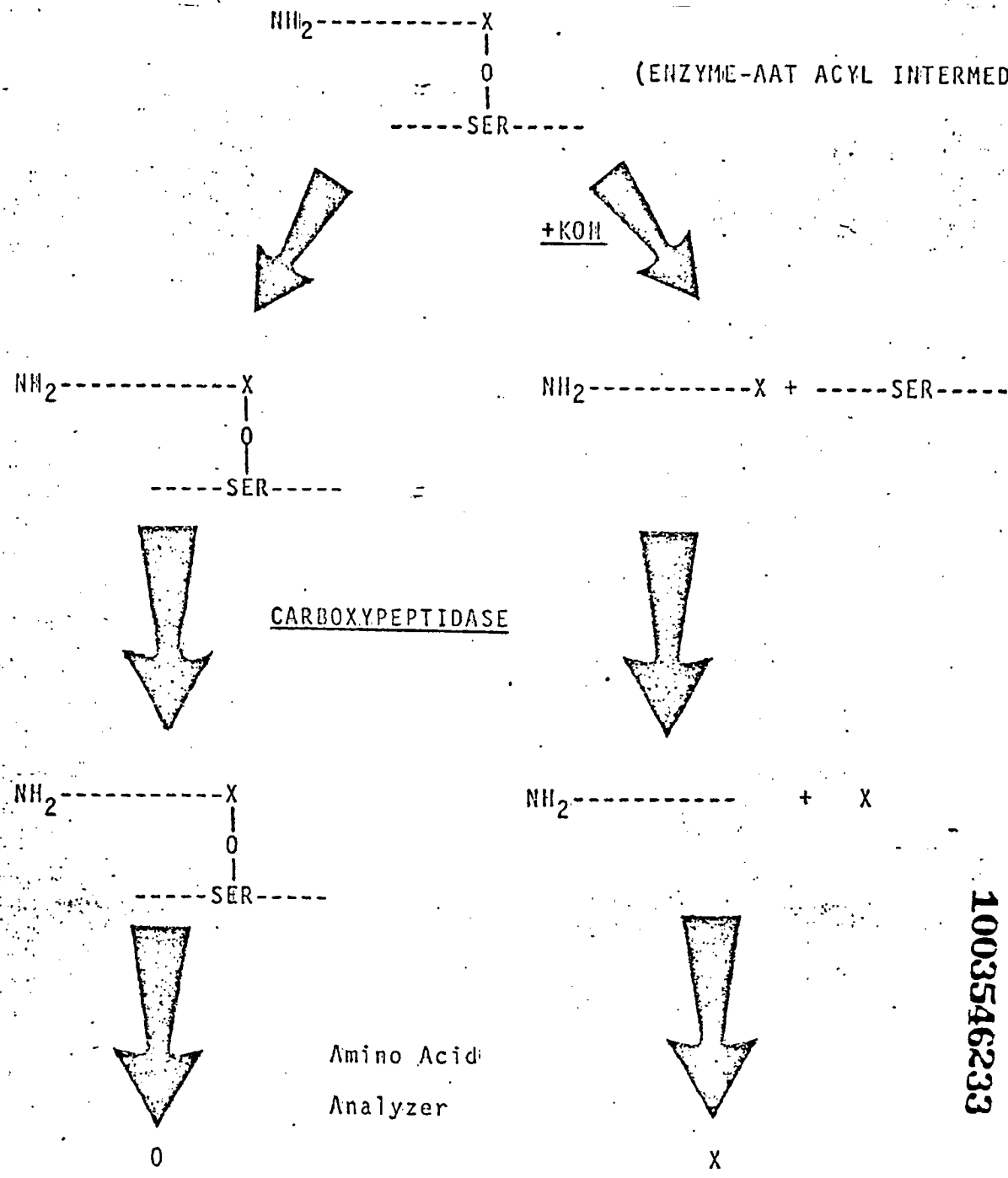


Figure. 10-2: Diagram of the Hypothesized Stages of Interaction of AAT with Enzymes.
SER= the serine amino acid at the catalytic site of the enzyme.
X = the amino acid at the inhibitory site of AAT.



10035462 33

Fig. 10-3: Diagram of Experiment Designed to Determine if Enzymes are Bound to AAT at the Carboxyl Terminal Amino Acid of AAT and if They are, to Determine the Identity of the Amino Acid (X) at The Inhibitory Site of AAT.

CTR Grant #954

Progress Report #1

Allen B. Cohen, M.D., Ph.D.
University of California, San Francisco
C/O Chest Division
San Francisco General Hospital
San Francisco, California 94110

May 1, 1974 to
January 15, 1975

THE GENETIC DEFECT IN ALPHA-1-ANTITRYPSIN IN DEFICIENT PATIENTS

The Interaction Between Alpha-1-antitrypsin (AAT) and Elastase:

The rationale for our original study of the elastase affinity column is outlined under item 6 of the renewal application. Briefly, an elastase affinity column seemed to be a logical method for attempting to isolate AAT of ZZ phenotype. However, examination of the products which are washed from an elastase column when AAT is added to it revealed that the AAT had lost its enzyme inhibitory activity and had lost a peptide which comprised less than 10% of its molecular weight. These experiments are reviewed below. The results seem to offer an unique opportunity to study some of the products of the interaction between AAT and elastase and perhaps to identify the key amino acid at the elastase inhibitory site of AAT.

Methods and Results:

The reactions of AAT with both soluble and sepharose-bound elastase were examined. The concentrations of enzymes and inhibitor were determined employing the extinction coefficient of individual protein. The catalytic activities of trypsin and elastase in hydrolyzing p-Toluenesulfonyl-L-Arginine Methyl Ester (TAME) and N-Acetyl-L-alanyl-L-alanyl-Lalanyl Methyl Ester (AAME), respectively, were measured with a Radiometer pH Stat as described by Gertler and Hofmann (1). Trypsin activity was also analyzed by the method of Erlanger, et. al. (2), using N-Benzoyl-DL-Arginine-p-Nitroanilide (BAPNA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the procedure of Laemmle (3) as modified by Hokin and co-workers (4). Bovine elastase (76 mg) was coupled to Cyanogen Bromide-activated Sepharose-4B (50 ml) following directions given by Pharmacia Fine Chemicals. The Sepharose-bound elastase hydrolyzed AMEE. A column packed with Sepharose-bound elastase was equilibrated with 0.1M Tris, pH 7.6 containing 0.5M NaCl. Purified and active AAT (20 mg) in the same buffer was applied to the column and two protein containing peaks were washed from the column (Fig. 1). The protein in the first peak showed a precipitin band with antisera against AAT in immunodiffusion analysis. However, it no longer inhibited the activity of trypsin when assayed with TAME and BAPNA or of elastase when assayed with AAME. The molecular weights of active and inactive AAT were estimated to be 54,000 and 50,000 daltons, respectively, by SDS-PAGE (gels 1 and 2 of Fig. 2). In addition, the reduced and non-reduced inactive AAT had identical mobilities (gels 2 and 4 of Fig. 2). The protein in the second peak passed through a molecular sieving filter which retains molecules in excess of 10,000 daltons, and it showed a single band in SDS-PAGE. When the column was disassembled and the Sepharose-bound elastase incubated with the irreversible elastase inhibitor phenyl methane sulfonyl fluoride, additional immunologically identifiable inactive AAT was found in a filtrate suggesting that AAT is a tightly

bound competitive inhibitor of elastase. Soluble elastase was incubated with AAT in 0.1M Tris, pH 7.6 at a molar ratio of approximately 1:2 (Elastase:AAT). At 1 min. and 60 min., aliquots were withdrawn from the incubation mixture and mixed with an SDS solution. The SDS-PAGE patterns of the 1 min. and 60 min. reaction mixtures showed protein bands with molecular weights of 73,500, 56,900, 54,000, 50,500 and 26,000 daltons. The 54,000 and 50,500 dalton proteins migrate identically with the active AAT applied to the elastase column and the inactive AAT eluted from the elastase column, respectively. The reactants in these gels, therefore, behave similarly to those on the elastase affinity column. These data imply that even in elastase excess the largest degradation which active AAT undergoes is the loss of one or more peptides with a molecular weight of about 3,500 daltons.

Significance:

The observed phenomena support the hypothesis that upon reaction of elastase with active AAT, an enzyme-inhibitor complex was formed at a 1:1 molar ratio. The complex dissociates into the enzyme and a modified form of AAT which has lost a peptide of less than 10% of its original weight. The modified AAT may have been associated with elastase through the carboxy-terminal amino acid of the modified AAT. (See item 10 of renewal application for discussion of this concept.) If this contention can be proven, we can identify the key amino acid at the inhibitory site of elastase on AAT. Since all enzymes inhibited by this site will cleave proteins at this amino acid one might begin the design of drugs to replace AAT in deficient patients with this amino acid and add appropriate inhibiting groups. There are already at least two such methods for tailoring inhibitors of serine proteases to the specificity of the enzyme (5,6).

Studies of the Trypsin Inhibitory Site:

The evolution of the products which develop during the interaction of trypsin and AAT was studied with cellulose acetate membrane (CAM), polyacrylamide gel, and SDS polyacrylamide gel (SDS-PAGE) electrophoretic techniques. We have confirmed Johnson's (7) and Moroi's (8) observations that when trypsin combines with an excess of AAT a single complex (Complex 1) forms and when it combines with an excess of trypsin, two complexes form (Complexes 1 and 2). The second complex has a molecular weight and electrophoretic mobility between the first complex and AAT (Fig. 3). We isolated the first complex on sephadex columns and demonstrated its identity because it contained the radio-labeled trypsin, it reacted against antisera to AAT, and it migrated like the first complex in an electrophoretic field (Fig. 4). Amino acid analysis (Table 1) and molecular weight determination supported Moroi's suggestion that trypsin and AAT combine in a 1:1 ratio but refuted Johnson's suggestion of 2:1 molar binding.

If the AAT-trypsin complex were connected by an ester bond to the serine hydroxyl in trypsin, the bond might be susceptible to degradation in alkaline pH. Treatment of the AAT-trypsin complex with KOH, pH 9.5 for 24 hours at 37°C results in the liberation of active trypsin and AAT with a slightly reduced molecular weight (Fig. 5) and more acidic charge (Fig. 6), suggesting that the AAT-trypsin bond is indeed broken in alkaline solutions. In addition the electrohporetic studies suggest

that a basic peptide of about 4,000 molecular weight may be lost during the interaction of trypsin and AAT. The amino acid analysis of the isolated complex is deficient in arginine, suggesting that the lost peptide may be basic because of a disproportionate number of arginine residues. These data are all consistent with the hypothesis that the trypsin-AAT complex is an acyl intermediate analogous to the acyl intermediate which serine proteases form with their substrates; however, with AAT the intermediate is stable and does not break down into products (Fig. 10-1 and 10-2, Item 10 of renewal application).

Purification of AAT of ZZ Phenotype by Antibody Affinity Chromatography:

One of the methods proposed to purify AAT of ZZ phenotype was antibody affinity chromatography (p. 11 of grant application). It was suggested that the AAT would have to be eluted with kaotropic ions like thiocyanate (9) and that hydrogen ion elution may not be used because of the pH lability of AAT. However, we have recently determined that AAT has a zone of stability at pH about 2.0 - 2.7 in HCl - KCl, or in glycine - HCl buffers (Table 2). These data now raise the possibility that AAT of ZZ phenotype could be purified with an antibody affinity column and eluted with one of these buffers.

References

1. Gertler, A. and Hofmann, T.: Can. J. Biochem. 48:384 (1970).

2. Erlanger, B.F., Kokowsky, N., and Cohen, W.: Arch. Biochem. 95:271 (1961).

3. Laemmle, U.V.: Nature 227:680 (1970).

4. Hokin, L.E., Dahl, J.L., Deupree, J.D., Dixon, J.F., Hackney, J.F., and Perdue, J.F.: J. Bio. Chem. 248:2593 (1973).

5. Thompson, R.C.: Biochem. 12:47 (1973).

6. Thompson, R.C. and Blout. E.: Biochem. 12:51 (1973).

7. Johnson, D.A., Pannell, R.N., and Travis, J.: Biochem. Biophys. Res. Commun. 57:584 (1974).

8. Moroi, M., and Yamasaki, M.: Biochem. Biophys. Acta. 359:130, 1974.

9. Dandliker, W.B., and DeSaussure, V.: Immunochem. LL:139, 1967.

TABLE 1

Alpha-1-antitrypsin Residues 52000 g		% Moles of Each Amino Acid/Mole Complex[a]			
		Measured	Calculated for 1:1 Molar Complex	Calculated for 1:2 Molar Complex[b]	Complex Predicted 1:X[c]
Lys[d]	34.01	7.60	6.92	6.35	1
His	12.81	2.74	2.49	2.22	1
Arg	9.31	0.00	2.00	1.88	2
Asp[e]	47.89	12.02	11.66	11.73	2
Thr[d]	29.85	6.72	6.24	5.81	1
Ser[d]	24.25	7.73	7.73	8.61	1
Glu	59.12	11.95	12.20	11.27	1
Pro	18.46	4.41	4.31	4.25	1
Gly[d]	26.27	8.32	8.24	9.12	1
Ala[d]	28.80	6.97	7.08	7.18	1
1/2 Cys	2.81	2.28	2.99	3.90	1
Val[d]	26.93	7.20	6.46	6.46	--
Met	7.96	1.40	1.48	1.29	1
Ile	15.62	5.10	4.45	3.66	1
Leu[e]	50.10	10.83	10.50	9.79	1
Tyr	5.84	1.85	2.19	2.56	1
Phe	25.22	5.17	4.58	3.90	1
Trp	not determined	--	--	--	--

Footnotes to Table 1

a % moles of each amino acid/mole complex was calculated from an amino acid analysis of aliquots of alpha-1-antitrypsin and trypsin which were used to form the complex

b 1:2 complex - 1 mole alpha-1-antitrypsin:2moles of trypsin

c complex predicted on the basis of whether the measured ratio of the amino acid was closer to the ratio predicted for a 1:1 or 1:2 complex. X = moles of trypsin.

d,e residues with greater than 15 and 20 residues difference between 1:1 and 1:2 molar complexes respectively. Therefore these residues would be more likely accurate predictors of the true ratio.

Legend to Table 1

These data show that ratios of 15 of the 17 measured amino acids in the alpha-1-antitrypsin-trypsin complex predict a 1:1 molar ratio. Three residues predicted a 1:2 molar ratio. These data suggest a 1:1 molar complex between alpha-1-antitrypsin and trypsin. The complex has been shown, however, to have lost a peptide of alpha-1-antitrypsin. The two residues which showed a 2:1 molar ratio may have had a disproportionately high ratio in the missing peptide. This formulation would predict that the peptide should be more basic than AAT and that the AAT peptide remaining in the complex should be proportionately more acidic. The increased acidic nature in AAT has in fact been shown by breaking the complex with KOH (Fig. 6). This suggestion is further supported because asp and gly should be the most accurate determinants of molar ratio because they each differ in the predicted 1:1 and 1:2 molar complexes by more than any other residue (27 residues each). This data therefore supports the data from SDS gel electrophoresis which suggests that the peptide has already been lost from the AAT-trypsin complex seen in various electrophoretic techniques and that the peptide which is lost has a peptide with a low proportion of aspartic acid relative to native alpha-1-antitrypsin. and a high proportion of arginine.

TABLE 2

pH.	2.0	3.0	4.0	5.0	6.0	7.4	9.0	10.0	11.0	12.0
2 hr.	26.4	39.0	39.2	26.0	23.8	25.5	24.2	23.8	23.4	24.9
4 hr.	24.8	35.7	33.8	23.4	22.5	24.8	24.8	25.5	25.7	26.6
6 hr.	25.7	39.8	39.2	25.7	25.7	26.6	26.0	24.1	25.8	26.2
8 hr.	25.4	38.4	39.6	27.3	23.8	22.8	23.9	24.1	23.6	25.3
24 hr.	27.8	42.4	41.9	27.3	26.0	28.9	27.0	27.0	27.9	26.6

Standard Curve Day I

2 µg 6.6
5 µg 19.6
10 µg 40.6

Standard Curve Day II

2 µg 8.7
5 µg 19.0
10 µg 38.5

pH Stability of Alpha-1-antitrypsin.

Lyophilized, purified AAT was placed in low ionic strength buffer at the specified pH and incubated at 25°C for the specified time. The pH was then raised to 7.6, 10 µg of trypsin were added and the trypsin activity was measured. Results are expressed as moles of substrate hydrolyzed/min. X 10^7. Higher enzyme rates indicate low inhibitory activity.

10. Plan for the Coming Year:

From the work in our laboratory during the past year, a hypothesis can be proposed for the biochemical mechanism of AAT and the hypothesis can be subjected to experimental tests. Earlier, we proposed that AAT interacted with enzymes in a manner similar to their interaction with protein substrates but instead of cleaving the substrate, AAT and the enzyme formed a stable intermediate. The interaction of serine proteases and substrates has been schematized by Stroud (Fig. 10-1). The schematic shows the different intermediates which develop during the interaction of serine proteases with substrates. Recently, Moroi (Biochem. Biophys. Acta. 359:130, 1974) has suggested that the AAT-enzyme complex is one of these intermediates, called the acyl intermediate (Panel 4, Fig. 10-1). In this intermediate the peptide on the amino-group side of the peptide cleavate has fallen from the complex and the serine hydroxyl at the enzyme catalytic site is bound to the carboxyl group of the amino acid at the end of the remaining protein fragment. This bond is an ester bond, and as mentioned in the progress report these bonds are often susceptible to cleavage under mild alkaline conditions. Our work in the past year has shown that the AAT in the AAT-enzyme complex has already lost a peptide and that the loss of the peptide is not dependent on unbound or active trypsin in the solution. In addition, the bond between the AAT and trypsin is broken under mild alkaline conditions. These data are highly suggestive that the AAT-enzyme complex is indeed an acyl intermediate (Fig. 10-2).

During the ensuing year we wish to test the next step in the hypothesis: Enzymes are attached by an alkaline labile bond to the new carboxy terminal amino acid on AAT. The design of the experiments are diagramed in Figure 10-3.

We have already determined that KOH can cleave the AAT-trypsin complex into an active enzyme and a fragment of AAT with a small peptide missing. If the enzyme is bound to the carboxy-terminal amino acid through its carboxyl group, as it must be to fulfill the requirements of the hypothesis, then the AAT in the complex will have no chemically recognizable carboxy-terminal amino acid. Carboxypeptidases are enzymes which cleave amino acids from the carboxy-terminal end of proteins, one residue at a time. Therefore, if the AAT-enzyme complex is an acyl intermediate and if a carboxypeptidase of appropriate specificity is added to the complex, the carboxy-terminal amino acid of the enzyme-bound fragment of AAT will not be removed (Fig. 10-3, left arrows). However, if the complex if first dissociated and then the carboxypeptidase is added, the amino acid at the enzyme inhibitory site will be removed and determined in the amino acid analyzer (Fig. 10-3 right arrows).

The experiment can be further illustrated by describing its use in determining the amino acid at the inhibitory site on AAT for trypsin. Carboxypeptidase B (CPB) is an enzyme which cleaves arginine and lysine amino acids from the carboxy-terminal end of proteins and trypsin cleaves proteins at arginine and lysine residues inside of the peptide chain. Therefore, a solution containing AAT-trypsin complexes will be divided into two aliquots and one aliquot will be dissociated with KOH. Then both aliquots will be treated with CPB. Amino acids which are released will be analyzed on an amino acid analyzer. If trypsin is bound to carboxy-terminal arginine or lysine on the remaining AAT peptide, then an arginine or lysine should be removed only from the complex dissociated in KOH. This experiment would have the dual function of demonstrating that trypsin is bound to the carboxy-terminal amino acid of the remaining peptide, and identifying this key amino acid. All enzymes inhibited by this

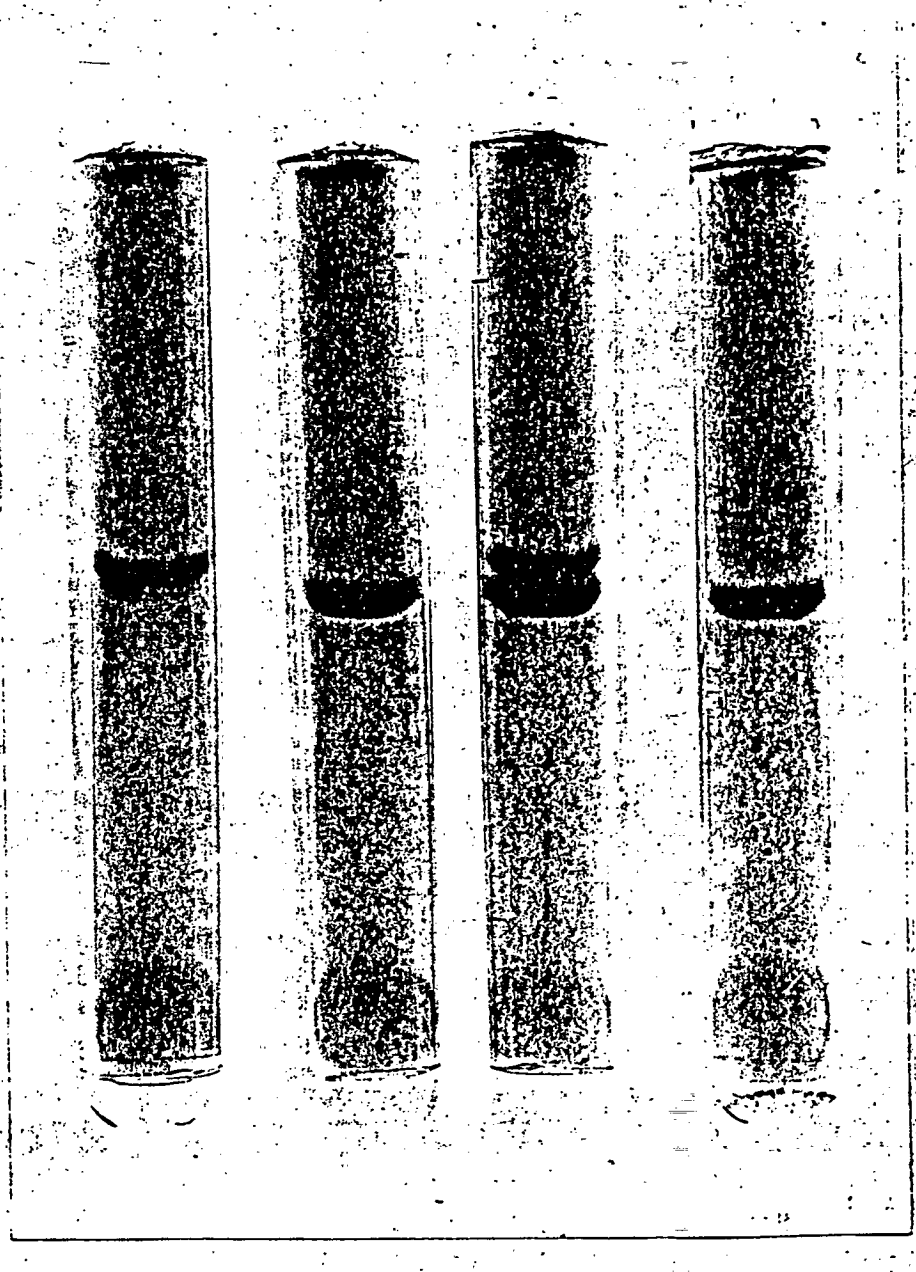


Figure 2. The SDS-gel electrophoretic patterns of active AAT (reduced; gel 1), inactive AAT (reduced; gel 2), mixture of active and inactive AAT (reduced; gel 3), and inactive AAT (non-reduced; gel 4). The separating gel was 9% total acrylamide. The stacking gel was 3.08% total acrylamide. The electrode buffer was tris-gly, pH8.3 containing 0.1% SDS. Electrophoresis was conducted at room temperature for 4 hours. A constant current of 3.0 ma per gel was applied. Migration is from top (Cathode) to bottom (Anode).

Figure 3 A:
Cellulose Acetate Membrane Electrophorogram of AAT and Trypsin: A. AAT is in molar excess of trypsin. B. Trypsin is in molar excess of AAT. C. Native AAT control. A black string is stretched below the colplex formed in the presence of AAT excess and above the complex formed in trypsin excess. Trypsin has migrated off the strip in an anodal direction. (The anode is at the bottom and the cathode is at the top.)

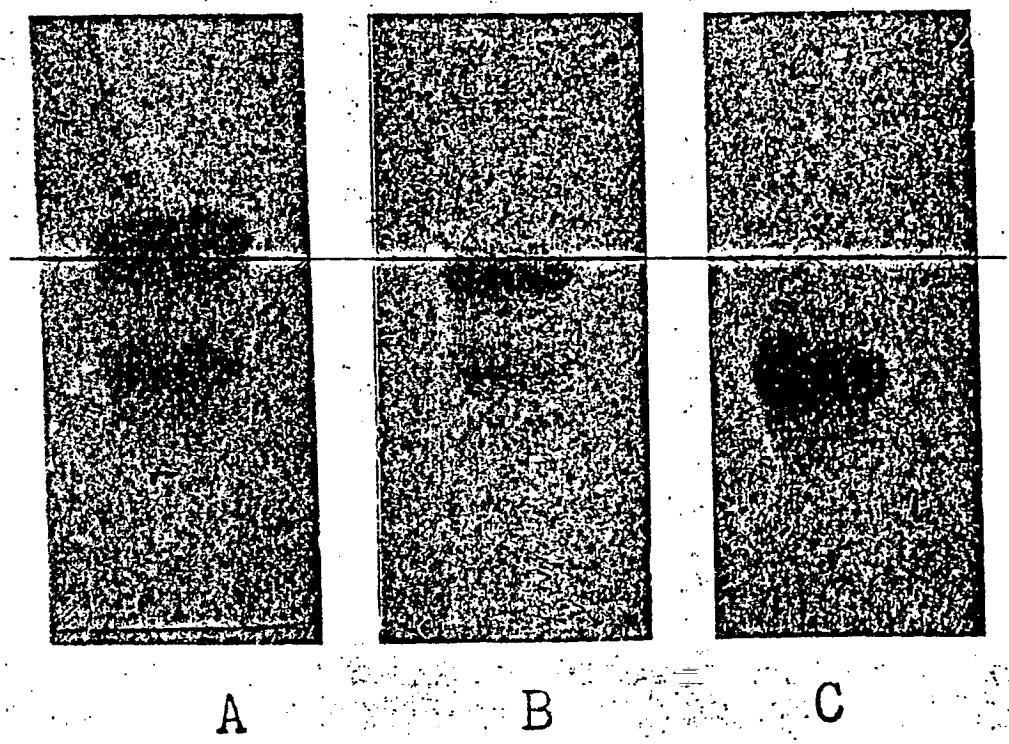


Figure 3 B:
Sodium Dodecyl Sulfate-Poyacrylamide Gel Electrophorogram of the Interaction of AAT and Trypsin with a Molar Excess of Trypsin.

A. AAT and Trypsin were added to sodium dodecyl sulfate (SDS) before mixing them together. No complex forms and therefore, this gel serves to locate native AAT and Trypsin as controls.

B. Run after reactants incubated at room temperature for one minute. A 73,000 dalton complex is present.

C. Run after reactants incubated at room temperature for 30 minutes. In addition to the 73,000 dalton complex, an additional complex of 62,000 daltons has formed. This complex does not form when AAT is in molar excess and in the presence of trypsin inhititors added at one minute. It is therefore, dependant on free active trypsin to form it. Data indicate that it represents the 73,000 dalton complex minus a 12,000 dalton peptide ocleaved form the bound trypsin.

D. Run after reactants incubated at room temperature for 60 minutes. The 73,000 dalton complex has been completely converted into the 62,000 dalton complex.

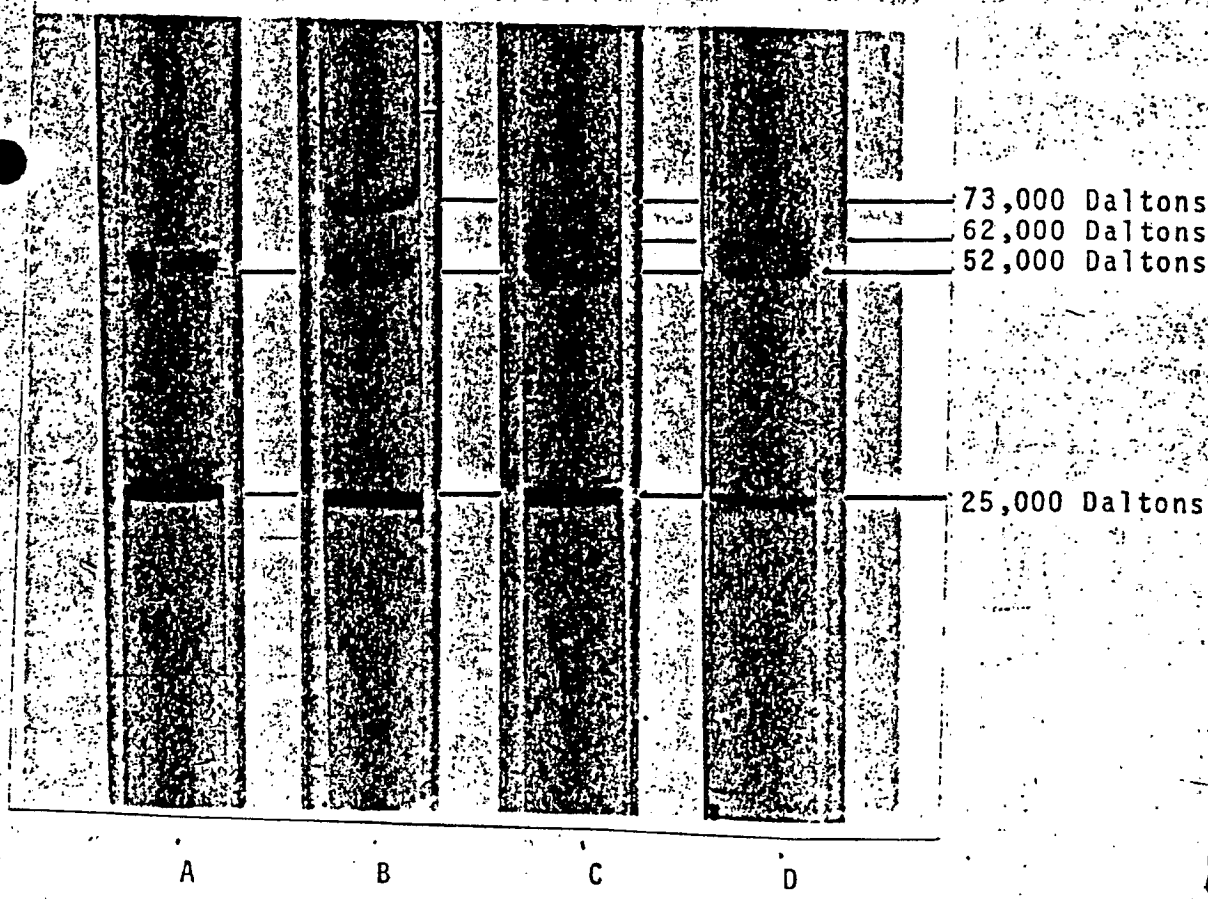
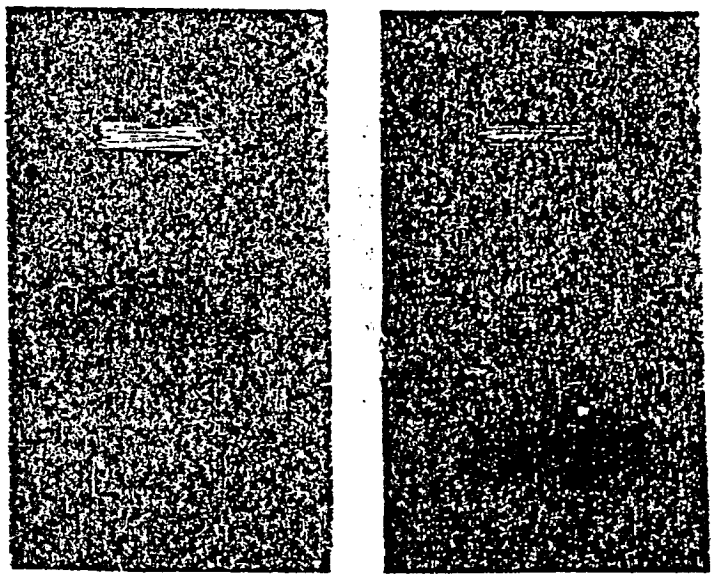


Figure 4:
Cellulose Acetate Membrane Electrophoregram of AAT-trypsin Complex Formed in AAT excess and Isolated on a Sephadex Column: A. Isolated complex. B. Native AAT control. The isolated complex contained radioactivity from the I-125 labeled trypsin and reacted in double-immunodiffusion of Ouchterlony to give a precipitin line between the isolated complex and native AAT.



A B

Figure 5:

Polyacrylamide Gel Electrophorogram in Sodium Dodecyl Sulfate to Study the Effects of KOH and Phenyl Methane Sulfonyl Fluoride (PMSF) on the Interaction between AAT and Trypsin.

In samples A-D there was a 2:1 molar excess of AAT:trypsin. The AAT and trypsin were combined and incubated for 1 minute. Then PMSF was added to samples A and B. After 15 minutes, the pH was raised to 9.5 with KOH in samples C and D. Sample E contained only fresh native AAT and Sample E contained only native trypsin. These SDS-PAGE showed that PMSF had no influence on complex formation (A). The 73,000 molecular weight complex forms when excess AAT is added to trypsin (D). When these reactants are incubated in KOH at 37°C for three hours, an additional complex (62,000 daltons) is seen (C). This complex has been shown to develop as a result of the cleavage of a large peptide from the bound trypsin by free trypsin; therefore, the KOH has released active trypsin into the reaction mixture. In addition, the AAT band is split, indicating that the released AAT has lost a small peptide of about 4000 daltons. Free trypsin is not seen in gel C because active trypsin incubated for several hours is broken down into peptides which are not recognizable on these gels. When KOH is added after PMSF the released trypsin is inactivated by the PMSF. Therefore, the 62,000 dalton complex is not seen and a trypsin band is seen. In addition, the low molecular weight AAT band is still present, showing that the appearance of this AAT cleavage is not dependant on the free active trypsin, but is the AAT which was released from the complex. These data suggest, therefore, that KOH has dissociated the complex into active trypsin and AAT which has lost a peptide of about 4000 daltons.

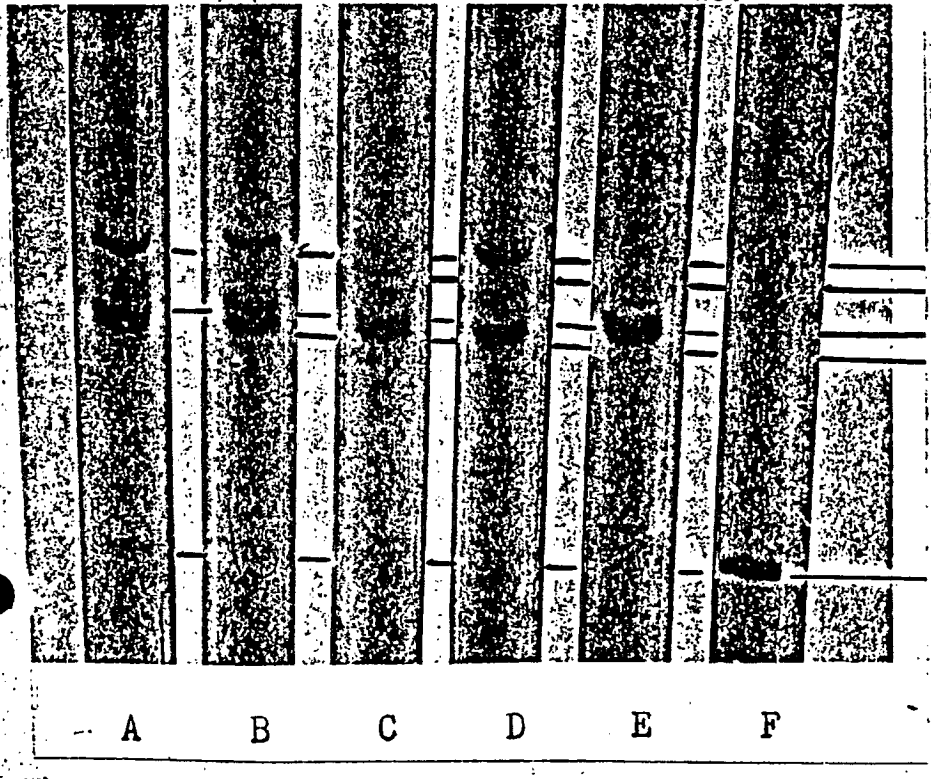


Figure 6:

Cellulose Acetate Electrophorogram to study the Effects of KOH and Phenyl Methane Sulfonyl Fluoride (PMSF) on the Interaction Between AAT and Trypsin.

In samples A-C there was a 2:1 molar excess of AAT:trypsin. The experiment was performed as described in Figure 3. Sample A contained PMSF and KOH, B contained KOH only, C did not have either KOH or PMSF. In electrophorogram "C" one band is formed by the interaction of AAT and trypsin. In electrophorogram "B" the complex band,seen at the top is split. This pattern is seen when excess free trypsin is present and therefore, indicates that free trypsin has been liberated by dissociating the AAT-trypsin complex. The splitting of the lower band indicates that AAT released from the complex is more acidic than native AAT (D) and has therefore, lost a peptide which is basic relative to native AAT. In electrophorogram "A",PMSF, a trypsin inhibitory, has prevented the appearance of the split band at the top,again, indicating that this band requires the presence of active trypsin. However, the AAT with more anodal migration is evident because of the persistence of active trypsin. However, the AAT with more anodal migration is is unaffected by the presence of PMSF as seen by the persistence of the split band at the bottom.

(The anode is at the bottom).